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PLURIPOTENTIAL CELLS-2

The invention herein described relates to isolated pluripotential cells, comprising at least part of the cytoplasm derived from an embryonic stem cell/embryonic germ cell and a nucleus of a somatic cell; methods to prepare such cells; therapeutic compositions of said cells; and uses thereof.

Animal embryonic development is a highly regulated development process that combines cell proliferation and cell/tissue differentiation to produce an intact organism. The co-ordination of cell proliferation and differentiation is, and has been, the subject of intense research and the information derived from this has contributed to our understanding of cell function and disease. For example and not by way of limitation, regulation of gene expression, cell differentiation, oncology, teratology.

Mammalian embryonic development is remarkably conserved during the early stages. Post fertilisation the early embryo completes four rounds of cleavage to form a morula of 16 cells. These cells complete several more rounds of division and develope into a blastocyst in which the cells can be divided into two distinct regions; the inner cell mass, which will form the embryo, and the trophectoderm, which will form extra embryonic tissue, (eg placenta).

Those cells that form part of the embryo up until the formation of the blastocyst are said to be totipotent (e.g. each cell has the developmental potential to form a complete embryo and all the cells required to support the growth and development of said embryo).

During the formation of the blastocyst, the cells that comprise the inner cell mass are said to be pluripotential (e.g. each cell has the developmental potential to form a variety of tissues).

Embryonic stem cells may be principally derived from two embryonic sources. Pluripotential cells isolated from the inner cell mass are termed embryonic stem cells (ES cells). An alternate source of pluripotential cells is derived from primordial germ cells isolated from the mesenteries or genital ridges of days 8.5-12.5 *post coitum* embryos which would ultimately differentiate into germ cells. These pluripotential cells are referred to as embryonic germ cells (EG cells). Each of these types of pluripotential cell has the same developmental potential with respect to differentiation into alternate cell types.

It is important to note that an intact embryo cannot be produced from a single pluripotential cell (eg either an ES or EG cell). Therefore a pluripotential cell has an increased commitment to terminal differentiation when compared to a totipotent cell.

For the sake of clarity where the term pluripotential cell is used it will refer equally to ES and/or EG cells.

The establishment of *in vitro* cultures of ES/EG cells has proven to be problematic. It has only recently be shown that *in vitro* cultures of ES/EG cells derived from non-murine species can be established (please see US 5 453 357 and US 5 690 926). Typically the ES/EG cultures have well defined characteristics. These include, but are not limited to;

i) maintenance in culture for at least 20 passages when maintained on fibroblast feeder layers;

ii) produce clusters of cells in culture referred to as embryoid bodies;

iii) ability to differentiate into multiple cell types in monolayer culture;

iv) can form embryo chimeras when mixed with an embryo host;

v) express ES/EG cell specific markers.

Until very recently, *in vitro* culture of human ES/EG cells was not possible. The first indication that conditions may be determined which could allow the establishment of human ES/EG cells in culture is described in WO 96/22362. The application describes cell lines and growth conditions which allow the continuous proliferation of primate ES cells which exhibit a range of characteristics or markers which are associated with stem cells having pluripotent characteristics.

For example, and not by way of limitation, the expression of specific cell surface markers SSEA-3 (+), SSEA-4 (+), TRA-1-60 (+), TRA-1-81 (+) (Shevinsky et al 1982; Kannagi et al 1983; Andrews et al 1984a) and alkaline phosphatase (+). In addition the established primate cell lines disclosed in WO 96/22362 have stable karyotypes and continue to proliferate in an undifferentiated state in continuous culture. The primate ES cell lines also retain the ability, throughout their continuous culture, to form tissues derived from all three embryonic germ layers (endoderm, mesoderm and ectoderm).

More recently Thomson *et al* 1998 have published conditions in which human ES cells can be established in culture. The above characteristics shown by primate ES cells are also shown by the human ES cell lines. In addition the human cell lines show high levels of telomerase activity, a characteristic of cells which show the ability to divide continuously in culture.

The establishment of human EG cell cultures is disclosed in WO 98/43679. This application describes the isolation of EG cells from the gonadal or genital ridges of human embryos. EG cells described in WO 98/43679 exhibit features in common with primate and human ES cells, (eg expression of cell surface markers, continuous proliferation in culture in an undifferentiated state, normal karyotype and the ability to differentiated into selected tissues under defined conditions).

It is evident that the use of *in vitro* cultures of pluripotential stem cells, especially human cells, has important ramifications for both basic research (eg as a model for studying gene expression and/or tissue differentiation) and in transplantation and/or replacement therapies for tissues which have been damaged either through injury or disease. The establishment of *in vitro* cultures of human ES and EG cells is a major step toward realising the full potential of this technology; because of their pluripotent nature ES and EG cells may be capable of differentiating under controlled conditions into a variety of cell types and/or tissues and organs that could have a wide variety of applications. For example, and not by way of limitation, replacement of damaged and/or diseased coronary and/or major arteries; replacement of damaged and/or diseased organs (eg as a result of kidney disease, (eg cirrohosis), diabetes, various autoimmune diseases); replacement of damaged neurones (eg Alzhiemers disease, Parkinsons disease, spinal injuries) or cancer. It will also be apparent to one skilled in the art that diseases such as AIDS may benefit from from tissues derived from ES or EG cells. The depletion of T-cells through virus induced cell death is the major contributory factor to the immuno-compromised state of AIDS suffers.

However, there are practical and ethical difficulties associated with the use of material derived from human embryos. Morever, such allogeneic material, if transplanted into another human, may illicit a severe immune reaction in the host and be thus destroyed.

It has been known for many years that amphibian somatic cell nuclei retain their ability to give rise to entire organisms when they are transplanted into egg cells which have had their nucleus removed or inactivated (Gurdon 1974). Thus determination of the pluripotent of these cells must be controlled by the egg cytoplasm which was able to in effect reprogramme the somatic cell nucleus into a totipotent state.

Mammalian somatic cell nuclei have also been shown to retain this placicity and can be reprogrammed when transferred to enucleated oocytes, (Campbell *et al* 1996; Wakayama *et al* 1998)

Moreover nucleated mouse ES cells have been shown to be able to reprogramme somatic cell nuclei, although in this case, a heterokaryon was produced containing the cytoplasm and nuclei from both types of cells so it is difficult to determine the actual mechanism of action of the reprogramming state.

In all these examples, althought the material produced is genetically identical to the somatic cell donor, these somatic cells were reprogrammed by cellular elements are derived from either ooctyes or ES cells and again, in human this poses practical and ethical concerns.

Methods that promote the fusion of cells are well known in the art (Kennett et al 1979). However, although it is relatively easy to fuse cells to form hybrid cells, nuclear fusion results in a cell containing two sets of chromosomes. This has enabled scientist to study the dominant expression of cell markers characteristic of each cell type and indeed enabled some to study mitotic chromosome stability in cross species hybrids. It is also well known in the art that cell hybrids may be formed by fusing the cytoplasm of a cell (in which the nucleus has been removed) with a selected intact cell to form a so called cybrid (Ege et al 1973; Veomett et al 1974; Wright and Hayflick et al 1975) This has enabled investigation into nucleo-cytoplasmic interactions and, in particular, the influence of cytoplasmic determinants on nuclear gene expression.

It has been known for several years that selected chemical treatments of cells in culture can result in cells extruding nuclei resulting in the formation of separate nuclear and cytoplasmic parts termed karyoplasts and cytoplasts, respectfully. These sub-cellular components have been used in fusion experiments. For example, and not by way of limitation, as mentioned, it is possible to produce a cytoplast from one cell and fuse the cytoplast to a selected cell to form a cytoplasmic hybrid or cybrid. In addition it is also possible to fuse the karyoplast or cell with a selected cell to form a nuclear hybrid. The nuclei fuse after nuclear membrane breakdown during mitosis and reconstitute after cytokinesis to form a polyploid or anueploid nucleus. The afore described techniques are well known in the art and will not be detailed extensively at this stage.

We have prepared cytoplasts, or parts thereof, derived from ES/EG cells and fused said cytoplasts with selected somatic cells to form cybrids. The aim of this approach is to re-programme the differentiated somatic cell nucleus

through contact with factors located in the ES/EG cytoplasm, so that the cybrid de-differentiates and so takes on the characteristic features of a pluripotential cell. This then provides the basis for the establishment of pluripotential cell lines which, upon exposure to various differentiation factors, can lead to the production of selected differentiated tissue for use, *inter alia*, transplantation therapy. The pluripotential cells so formed retain the nucleus of the somatic cell and at least part of the cytoplasm of the ES/EG cell (the mitochondrial genome would be retained and replicated by the cybrid). Ideally, the somatic nucleus is derived from a patient requiring transplant tissue so that the tissue produced by the aforementioned method is immunologically compatible with the patient requiring the transplant. The use of ES/EG cells directly in the production of tissue means the tissue is not entirely immunologically "silent" due to the presence of a complete set of male or female chromosomes from one of the parents of the embryo formed for the purpose of providing the ES/EG cells.

It is therefore an object of the invention to provide a pluripotential cell and corresponding cell line.

It is a further object of the invention to provide a differentiated tissue for use in transplantation therapy.

According to a first aspect of the invention there is provided a cell comprising at least part of the cytoplasm derived from at least one embryonal stem cell or embryonal germ cell combined with the at least the nucleus of at least one somatic cell.

In a preferred embodiment of the invention said cell, ideally a cybrid, is characterised by the possession of at least one pluripotential characteristic.

We believe that the acquisition of this pluripotential characteristic is as a result of the re-programming of said somatic nucleus.

It will be apparent to those skilled in the art that the cell of the invention may be derived, most preferably, by the creation of a cybrid; but an alternative option involves the fusion of a somatic cell with an ES/EG cell. Clearly this latter option is not preferred because subsequent mitosis will result in a hybrid having an abnormal karyotype.

Ideally said pluripotential characteristic includes the ability to differentiate into at least one selected tissue type, preferably upon exposure to at least one differentiation factor.

Alternatively, or additionally, said pluripotential characteristic includes the ability of said cell to proliferate in culture in an undifferentiated state.

In yet a further preferred embodiment of the invention said cell has the capacity to proliferate in continous culture in an undifferentiated state for at least 6 months and ideally 12 months.

Alternatively or additionally, said pluripotential characteristic includes the expression of at least one selected marker of pluripotential cells.

It is well known in the art that pluripotential cells express a number of genes not typically expressed by differentiated cells. These are valuable tools to monitor whether the ES/EG cytoplasm has re-programmed a somatic cell nucleus. One such example is Oct4.

In a preferred embodiment of the invention said selected marker is expression of the Oct4 gene.

In yet still a further preferred embodiment of the invention said selected marker is a cell surface marker. Preferably said cell surface marker is selected from the group including : SSEA-1 (-);and/or SSEA-3 (+); and/or SSEA-4 (+); and/or TRA-1-60 (+); and/or TRA-1-81 (+); and/or alkaline phosphatase (+).

Alternatively or additionally said pluripotential characteristic includes the presence of telomerase activity in said pluripotential cell. Ideally said telomerase activity is correlated with extension of telomeres.

For the sake of clarity, telomerase enzymes add, *de novo*, repetitive DNA sequences to the ends of chromosomes. These ends are referred to as telomeres. For example the telomeres of human chromosomes contain the sequence '5 TTAGGG 3' repeated approximately 1000 times at their ends. In young, dividing cells the telomeres are relatively long. In aging, or non-dividing cells, the telomeres become shortened and there is a strong correlation between telomere shortening and capacity to proliferate. Methods to increase the length of telomeres to increase proliferative capacity are known in the art and are described in WO9513383.

Alternatively or additionally said pluripotential characteristic includes the presence of a chromosomal methylation pattern characteristic of pluripotential cells.

It is well known in the art that the genome of eukaryotic organisms is variably methylated through the addition of methyl ($-CH_3$) groups attached to cytosine residues in DNA to form 5'methylcytosine (5'-mC). Methylation is correlated

with the control of gene expression. Typically genes that are hypomethylated tend to be highly expressed. Hypermethylation is correlated with reduced gene expression. It will be apparent to one skilled in the art that pluripotential cells will have a typical methylation pattern. This pattern may be analysed at a genomic level or at the level of a specific gene. Methods to analyse the extent of methylation are well known in the art and include, by example and not by way of limitation, restriction enzyme digestion of DNA with methylation sensitive restriction endonucleases followed by Southern blotting and probing with suitable gene probes (Umezawa et al 1997).

Alternatively or additionally said pluripotential characteristic includes the ability to induce tumours when introduced into an animal, ideally a rodent experimental model. More ideally still said animal is immunosupressed

According to a second aspect of the invention there is provided a cell-line comprising cells according to the invention. Ideally, said cell- line are of human origin.

According to a third aspect of the invention there is provided a method for preparing a cytoplast, or part thereof, for use in the production of the cell or cell line of the invention comprising;

i) providing at least one ES/EG cell;
ii) separating at least part of the cytoplasm from the nucleus of said ES/EG cell;
iii) isolating said cytoplasmic part; and, optionally
iv) storing said isolated cytoplasmic part prior to use.

In a preferred method of the invention said cytoplasmic part is a cytoplast.

It will be apparent to one skilled in the art that said cytoplast may be provided either as an aliquot isolated from at least one ES/EG cell (eg an aliquot extracted from an intact ES/EG cell via micromanipluation techniques) or alternatively and preferably, said cytoplasmic part may be provided as an isolated cytoplast.

In a preferred method of the invention said cytoplast is separated from said nucleus by exposure to a pharmacologically effective amount of a cytochalasin. Ideally, cytochalasin B.

It is well known in the art that cytochalasin B is an example of a chemical effective at separating the nucleus of a cell from the cytoplasm to form a karyoplast and cytoplast respectively, (Methods in Enzymology Vol 151, p221-237 1987).

According to a fourth aspect of the invention there is provided a method for preparing a cell or cell line in accordance with the invention comprising;

i) combining at least one ES/EG cell with at least one somatic cell;

ii) removing from said combined cell, the ES/EG cell nucleus;

iii) culturing said cell under conditions conducive to proliferation and expansion of said cell; and, optionally

iv) storing said cell culture under suitable storage conditions.

It will be apparent to one skilled in the art that methods of micromanipulation exist that facilitate the removal of nuclei from selected cells. It will be apparent that this method of the invention advantageously provides that ;

i) the factors produced by the ES/EG cell are continually produced thereby maintaining a steady-state level of factors necessary to reprogramme the somatic cell nucleus; and

ii) the ES/EG cell nucleus is removed from the combined cell prior to mitosis ensuring nuclear fusion does not occur.

It will be apparent to those skilled in the art that the nature of the somatic cell selected is not critical to the operation of the invention although the cell-type will be selected so as to optimise or maximise success in terms of production of a cell or cell-line of the invention.

According to a fifth aspect of the invention there is provided a method for preparing a cell or cell line in accordance with the invention comprising;

i) providing at least part of the cytoplasm of an ES/EG cell;

ii) combining said cytoplasmic part with at least one somatic cell;

iii) growing said combined cell in culture; and, optionally

iv) storing said combined cell under suitable storage conditions.

In a preferred method of the invention said cytoplasmic part is provided as a cytoplast.

In yet a further preferred method of the invention said cytoplast is combined with said somatic cell via cytoplast/somatic cell fusion.

In the above described methods the ES/EG cell and somatic cell are, ideally of human origin.

According to a sixth aspect of the invention there is provided a cell culture comprising at least one cell according to the invention.

According to a seventh aspect of the invention there is provided a method for inducing differentiation of at least one cell of the invention comprising:

i) providing a cell according to the invention;

ii) culturing said cell under conditions conducive to the differentiation of said cell into at least one tissue; and,optionally

iii) storing of said differentiated tissue prior to use under suitable storage conditions.

Ideally said culture conditions are selected from so as to provide a tissue type, by example and not by way of limitation, that is neuronal, muscle (eg smooth, striated, cardiac), bone, cartilage, liver, kidney, respiratory epithelium, haematopoietic cells, spleen, skin, stomach, intestine.

According to a eighth aspect of the invention there is provided at least one tissue type or organ comprising at least one cell according to the invention.

It will be apparent to one skilled in the art that differentiated tissue according to the invention may have extensive application with respect to transplantation therapy. For example, and not by way of limitation, replacement of damaged and/or diseased coronary and/or major arteries; replacement of damaged and/or diseased organs (eg as a result of kidney disease (cirrohosis), diabetes, various autoimmune diseases); replacement of damaged neurones (eg Alzhiemers disease, Parkinsons disease, spinal injuries), or cancer. It will also be apparent to one skilled in the art that diseases such as AIDS may benefit from from tissues derived from the cells of the invention. The depletion of T-cells through virus induced cell death is the major contributory factor to the immuno-compromised state of AIDS suffers. The provision of a non-

exhaustive supply of T-cells derived from a non-infected somatic cell from the patient has obvious benefits. Moreover, tissue rejection due to a host cell immune responses are likely to be negligible since the tissue is derived from the host into which the tissue is to be transplanted.

According to a nineth aspect of the invention there is provided a therapeutic composition comprising at least one cell of the invention including a suitable excipient, diluant or carrier.

In a preferred embodiment of the invention said therapeutic composition is provided for use in tissue transplantation.

According to a tenth aspect of the invention there is provided a method to treat conditions or diseases requiring transplantation of tissue comprising;

i) providing at least one tissue type or organ according to the invention;
ii) surgically introducing said tissue or organ into a patient to be treated;
iii) treating said patient under conditions which are conducive to the acceptance of said transplanted tissue by said patient.

According to an eleventh aspect of the invention there is provided a kit comprising; at least one cell according to the invention; instructions with respect to the maintenance of said cell in culture; and, optionally, factors required to induce differentiation of said cell to at least one desired tissue type or organ.

Embodiments of the invention will now be described, by example only and with reference to the following materials and methods and Figure.

Figure 1 shows PCR amplification of Oct4 mRNA from a human EC x somatic cell (thymocyte) heterokaryon.

Materials and Methods

This experiment exploits human tetratocarcinoma (EC) cells fused to mouse thymocytes. We reasoned that EC cells have many of the properties of ES/EG cells and are therefore a useful tool to analyse re-programming of somatic cell nuclei.

Preparation of Mouse Thymocytes

The thymocytes were obtained by mincing a thymus removed from a 4-6 week old male mouse (Swiss strain) and suspending the released cells in 10 ml medium (DMEM) with 10% foetal calf serum (FCS). After standing for 2-3 minutes to allow large fragments of thymus to settle, the supernatant was removed and centrifuged at 1500 rpm for 5 min to pellet the suspended thymocytes. The thymocytes were resuspended in fresh medium without FCS, and pelletted again by centrifugation; this was repeated a second time after which the cells were resuspended in fresh serum free medium and counted. Human EC cells were obtained by trypsinisation of confluent cultures as previously described (Andrews *et al.*, 1980; 1982). After washing two times in serum free DMEM, and counting, the human EC cells were mixed with the mouse thymocytes in a ratio of 1 EC cell to 10 thymocytes. The mixed cells were pelletted by centrifugation at 1500 rpm for 5 min.

Heterokaryon Fusion of Human EC cells and Mouse Thymocytes & Extraction of RNA

The cells were fused using polyethylene glycol (PEG) (Kennett, 1979). The pellet (in Experiment 1, 2 x 10^6 EC cells and 2 x 10^7 thymocytes; in Experiment 2, 3 x 10^6 EC cells and 3 x 10^7 thymocytes) was resuspended in

200 µl 50% (w/v) PEG 1500 in 75 mM HEPES, pH8.0 (Boehringer Mannheim) and incubated at 37° C for 1.5 min. Serum free medium, pre-warmed to 37° C, was then added gradually over 5 min. The cells were then pelletted by centrifugation at 1500 rpm for 5 min. and resuspended in 5 ml DMEM with 20% foetal calf serum. These cell were then plated into a T25 flask and placed in a humidified incubator (10% CO_2 in air) at 37°C for 2 days.

After 2 days, the non-attached cells were aspirated. The remaining attached cells were harvested by trypsinisation, and washed two times in DEPC-treated PBS to remove the serum. The pellet was then resuspended into Tri reagent (1 ml) to isolate RNA (Sigma-Aldrich Chemical Co., as described in Sigma Technical Bulletin MB-205). The isolated RNA was quantified by optical density measurements and the absence of contaminating DNA was determined by PCR using β-actin and HPRT primers in separate samples (Wakeman *et al.*, 1998). If free of DNA, the RNA was then used for RT.PCR analysis of Oct4 expression.

PCR Amplification of Oct4 from Human EC x Mouse Thymocyte Heterokaryon

In one experiment (2102Ep with thymocytes), a control was prepared, consisting of cells treated as for fusion except that the incubation with PEG was omitted - thus it was anticipated that no 2102Ep x thymocyte heterokaryons would be formed. In another experiment RNA was isolated from thymocytes alone and also from a mouse EC line (PCC4 aza1, clone 3), to provide further negative and positive controls for mouse Oct4 expression. cDNA was then produced from the samples using reverse transcriptase (RT) (Wakeman *et al.*, 1998). PCR was then performed using oligonucleotide primers specific for human and mouse *Oct 4*, a marker of pluripotent cells under the standard PCR conditions described in Wakeman *et al.* (1998) with

an annealing temperature of 61°C. These products were then subjected to electrophoresis and separated DNA fragments detected by ethidium bromide staining (Figure 7). Molecular size of the amplified fragments was determined by using a 1kb DNA step ladder.

PCR Primers for human and mouse Oct 4

Species	Annealing Temp (°C)	Sequence	Bp	GenBank Accession No. and primer location
Human Forward Reverse	61.4	5'-cgaccatctgccgctttgag-3' 3'-ccccctgtcccccattccta-5'	573	*X52437* 120-139 534-515
Mouse Forward Reverse	60.4	5'-gtccgcccgcatacgagttc-3' 3'-aggggccgcagcttacacat-3'	415	*Z11899* 361-380 937-918

These primers were designed using the PrimerSelect module of the Lasergene suite of programs (DNAStar Inc., USA). The mouse primers would not be expected to amplify human Oct4.

Enucleation of cells to yield 'cytoplasts' and 'karyoplasts' or 'mini-cells'.

One of the techniques that is employed in our method for producing Re-programmed Embryonic Stem cells (RPES cells) is the use of cytochalasin B to generate enucleated ES/EG cells (ES/EG cytoplasts) as the cytoplasm donor, and 'karyoplasts' (also called 'mini-cells') from the differentiated or committed cells as the nucleus donor. Cytochalasin B is well-known to induce cells to extrude their nuclei (Carter, 1967) and has been employed by numerous authors to induce enucleation of a wide range of cells of a variety of species including both mouse and human cells (Poste 1972; Prescott et al 1972; Goldman et al 1973; Wright and Hayflick 1973; Ege and Ringertz 1974a; Wigler and Weinstein 1975). Such enucleation results in a cell lacking a nucleus, but is otherwise intact and viable for a number of days (Goldman et al 1973); these enucleated cells have been called anucleate cells (Poste 1972) or cytoplasts (Veomett et al 1974). The nucleus that is extruded from the cell

retains a thin rim of cytoplasm and is surrounded by a plasma membrane; these structures have been called 'karyoplasts' (Veomett et al 1974) or 'mini-cells' (Ege and Ringertz 1975). Enucleation of cells to yield both cytoplasts and karyoplasts may be achieved by well-established techniques in which cells growing attached to a plastic disc are inverted over a solution of cytochalasin B in a centrifuge tube and centrifuged; the cytoplasts remain attached to the plastic disc, while the karyoplasts are pelleted at the bottom of the centrifuge tube (Prescott et al 1972). Alternatively, cells in suspension may be centrifuged through a density gradient, typically composed of Ficoll, containing cytochalasin B (Wigler and Weinstein 1975). In this case, cytoplasts and karyoplasts are formed and may be recovered from different parts of the gradient after centrifugation.

Methods for combining (fusing) the cytoplasm of one cell with the nucleus of another.

The methods for creating hybrid cells by fusing two or more cells of different origins together are very well established and widely known. For a review of the commonly used methods based upon Sendai virus induced cell fusion, or cell fusion induced by polyethylene glycol (PEG), see Kennett (1979). Briefly, mixtures of cells that it is desired to fuse are incubated with a fusogenic agent, such as Sendai virus or PEG, often with centrifugation or agitation to encourage clumping and close apposition of the cell membranes; variables such as time, temperature, cell concentration and fusogenic agent concentration are optimised for each cell combination. These techniques have also been shown to allow fusion of cytoplasts, prepared by cytochalasin B induced enucleation, with whole cells or karyoplasts, also derived by cytochalasin B induced enucleation (Poste and Reeve 1971; Ege and Ringertz

1975; Ege et al 1973, 1974; Veomett et al 1974; Wright and Hayflick 1975; Shay 1977)).

Another technique that is now well established and widely used for inducing cell fusion, 'electrofusion', involves passing short electric pulses through mixtures of cells (Neil and Zimmermann 1993).

Production of RPES cells

The production of RPES cells requires several steps:

1. the selection of appropriate differentiated cells (the Nucleus Donor) and, if necessary, the isolation of their nuclei,
2. the selection of ES/EG cells (the Cytoplasm Donor),
3. the fusion of the differentiated cell nuclei with the ES/EG cells, and
4. the removal of the ES/EG cell nucleus, either before or after fusion.

The production technique may, in some cases, be optimised by pre-treatment of the differentiated cells, or contemporaneous treatment of the differentiated cell/ ES/EG cell fused products, with various agents such as, but not limited to, inhibitors of DNA methylation, to enhance the ability of the differentiated cell nucleus to be re-programmed. After the production of the RPES cells additional methods are required to propagate the cells, to characterise their properties and to induce them to differentiate into required somatic cell types.

Differentiated cells to be used as Nuclear Donors

— A large range of somatic cells derived from any tissue or organ of an adult mammal or human, or from embryos or foetuses, or from extra-embryonic tissues such as the trophoblast or yolk sac may be used as a source of nuclei for reprogramming. Particular somatic cell types include but are not limited to thymocytes, peripheral blood lymphocytes, epidermal cells such as from the bucal cavity, cumulus cells, or other stem cells isolated from biopsies of various tissues, such as the bone marrow, the nervous system and the gut. The technique may also be applied to various established cell lines, such as those derived from various tumours including, for example, but not limited to lymphoblastoid cell lines. The selected somatic cells used for the re-programming procedure may be used directly upon isolation or they may be cultured for a short time before further manipulation. In some instances such somatic cells may be combined entirely with ES/EG cells as described below, or nuclei or karyoplasts may first be isolated from them, for example using agents such as cytochalasin B, as discussed above, or by other methods. For example, nuclei may also be isolated using established micromanipulation procedures, or other established cell fractionation procedures.

Fusion of parental differentiated cells and parental ES/EG cells to yield RPES cells:

Several methods may be used to combine the cytoplasm of an ES/EG cell and the nucleus of a differentiated cell to yield an RPES containing the nuclear genome of the differentiated cell but not the ES/EG cell.

A. Cells may be fused by use of chemical agents such as polyethylene glycol (PEG) or viruses such as Sendai virus, or by passing an electric current through a mixture of cells. As discussed above, these methods

are well known and may be readily applied. These methods may be used to fuse:

1. a differentiated cell with an ES/EG cell, or
2. a karyoplast from a differentiated cell with an ES/EG cell, or
3. a differentiated cell with one or more cytoplasts isolated from ES/EG cells, or
4. a karyoplast from a differentiated cell with one or more cytoplasts isolated from ES/EG cells.

In cases (1) and (2), the result will initially be a heterokaryon containing two nuclei, one from each parental cell. If this heterokaryon were allowed to divide the result would be a hybrid cell containing a single nucleus with a complete or partial genome from each parental cell. However, in our method of producing RPES cells, the ES/EG nucleus is removed prior to cell division of the hybrid cell, so that the derivative dividing cell population retains only the genome of the parental differentiated cell.

In cases (3) and (4) the ES/EG nucleus is removed from the ES/EG cell before fusion, for example by enucleation with cytochalasin B as discussed above, so that the resulting product contains only the differentiated cell nucleus and cytoplasm from the ES/EG cell parent. In any of these cases, the resulting RPES cells that continue to proliferate retain only the nuclear genome of the differentiated parental cell, which is now reprogrammed to express a new pattern of gene activity.

In cases (1) and (2) the ES/EG cell nucleus is removed from the heterokaryon in one of several ways that include, but are not limited to, partial enucleation using drugs such as cytochalasin B, applied in the same manner as described above for enucleating ES/EG cells and generating cytoplasts for fusion. In the present case in which enucleation is carried out after fusion, some heterokaryons lose both nuclei, in which case they do not proliferate, some heterokaryons lose the differentiated cell nucleus, in which case they retain the parental ES/EG nucleus and continue proliferating, some heterokaryons lose the ES/EG cell nucleus, in which case they continue proliferating as RPES cells, and some heterokaryons retain both nuclei and eventually continue proliferating as hybrid cells. Several methods are used to select the RPES cells and to eliminate any of the cells retaining an ES/EG cell genome or to eliminate any cells retaining a somatic nucleus that has failed to undergo re-programming. In one method, the proliferating cells are cloned by established techniques (e.g. by picking single cells with a micropipette - see Andrews et al 1982, 1984b), and individual clones are screened using genetic markers for those that retain an ES/EG genome. The latter cells are discarded, whereas those that retain only a differentiated cell genome but not an ES/EG cell derived genome, and express an RPES phenotype, are retained. Standard DNA genotyping techniques using well established DNA fingerprinting technology (Jeffreys et al 1985, 1988; Yan et al 1996) may be used to identify whether the nuclear genome of any proliferating cells is derived from either the ES/EG cell or differentiated cell parent, or both.

In another method, before use as a fusion partner, the ES/EG cell parent is genetically marked by insertion of a gene that will allow selection

against any cell carrying that gene; for example, the ES/EG cell can be stably transfected with a vector encoding the Herpes Simplex Virus-1 Tk gene (HSV1-Tk), such that any cells carrying that gene can be killed by culture in the presence of a number of drugs including acyclovir (9-[(2-hydroxyethoxy)methyl]guanine) or FIAU (1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil) (Borrelli et al 1988; Hasty et al 1991), or gancyclovir (Rubinstein et al 1993; McCarrick and Andrews 1992). In this method, following partial enucleation, the remaining heterokaryons are cultured in medium containing this drug, and only those that have lost the ES/EG cell nucleus survive. Other selectable genetic systems can also be similarly used. Persisting parental differentiated cells that have not been reprogrammed are removed by cloning the surviving cells, or by selecting RPES cells by virtue of their expression of specific surface antigen markers that include, but are not limited to, SSEA3, SSEA4, TRA-1-60 or TRA-1-81, as discussed above as characteristic markers of ES/EG cells. For the latter approach, fluorescence activated cell sorting (FACS), a widely used method for separating subsets of cells can be used (e.g. Andrews et al 1982, 1987; Ackerman et al 1994; Williams et al 1988).

In another method, the ES/EG cell parent is incubated prior to fusion, with a drug that irreversibly inactivates its nucleus and prevents its replication, for example, topoisomerase inhibitors such as etoposide (Downes et al 1991; Fulka and Moor 1993). The resulting heterokaryon naturally eliminates this treated nucleus prior to cell division, so that the resulting dividing cell population only contains the genome derived from the parental differentiated cell. This approach may also be combined with the preceding 'partial enucleation of heterokaryons' approach to ensure complete loss of the ES/EG genome.

– In another method, after cell fusion to produce a heterokaryon, the ES/EG cell nucleus is removed by micro-manipulation.

B. Rather than chemical, viral or electrically induced fusion, the nucleus of the differentiated cell is combined with an ES/EG cell parent by micro-manipulation. In this method, the nucleus of the differentiated cell is withdrawn using a micropipette inserted through the cell membrane. It is then injected either into an inoculated ES/EG cell, or into an intact ES/EG. In the later case the ES/EG cell nucleus is then removed by a similar technique, or by one of the techniques described above, before nuclear fusion and cell division occurs.

Growth and selection of RPES cells

Following fusion to combine a differentiated cell and an ES/EG cells, with prior or subsequent removal of the ES/EG cell nucleus, it is necessary to provide appropriate conditions for the re-programming of the differentiated cell nucleus and for the subsequent proliferation of the resulting RPES cells.

Several methods are used to enhance the efficiency of reprogramming:

1. prior to fusion the differentiated cell and ES/EG cell are synchronised with respect to position in the cell cycle, by use of reversible inhibitors that arrest the cell cycle at specific stages (e.g. nocodazole), or by the use of conditions such as low serum to arrest cells in G1, or by selection of cells at specific stages of the cell cycle by using vital DNA stains and flow microfluorimetry

(Fluorescence Activated Cell Sorting) (Ashihara and Baserga 1979; Andrews et al 1987; Crissman 1995; Stein et al 1995).

2. the differentiated cell or the immediate fusion product is cultured in the presence of drugs that inhibit methylation or promote demethylation (e.g. 5-azacytidine) (e.g. Taylor and Jones 1979; Jones 1985; Keshet et al 1986), or alter the structure of chromatin, for example butyrate, spermine, trichostatin A or trapoxin which inhibit deacetylation and promote acetylation of histones, which plays a role in X chromosome inactivation, gene imprinting and regulation of gene expression (Caldarera et al 1975; McKnight et al 1980; Stein et al 1997; Hu et al 1998; Wolffe and Pruss 1996;).

3. the period of time between production of heterokaryons and the removal of the ES/EG cell nucleus is made as long as possible without permitting nuclear fusion. This period can be elongated by culturing the heterokaryons under conditions that reversibly inhibit progress through the cell cycle (e.g. thymidine block - Stein et al 1995), or by altering growth conditions, such as serum starvation or lowered temperature, that retard cell division but permit reprogramming to proceed.

4. any, or all combinations of these methods.

In all these experiments the cells are cultured in standard cell culture media that include but are not restricted to Dulbecco's modified Eagle's Medium (DME, high glucose formulation) or Ham's F12, supplemented in some cases with foetal bovine serum or with other additives (e.g. see Andrews et al 1980, 1982, 1984, 1994). Subsequent to fusion and re-programming, the growth of the resulting cells may be optimised culture on feeder layers of cells that include, but are not restricted to, irradiated or mitomycin C treated STO cells,

or embryonic fibroblasts of various species, including humans (see Robertson 1987a; Thomson et al 1998). The cells may be cultured in the presence of various growth factors or other tissue culture additives, that include but are not restricted to LIF, FGF, SCF

Differentiation of the RPES cells

In the best cases, the RPES cells acquire pluripotent properties that closely resemble those of embryonic stem cells, so that the RPES cells are able to differentiate and to initiate differentiation pathways that result in the formation of any cell type that may be found in the adult, embryo or in extra-embryonic tissues, given appropriate conditions. The maintenance of an ES/EG cell state can be monitored by assay of various markers that include the cell surface antigens SSEA3, SSEA4, TRA-1-60, TRA-1-81, by their expression of alkaline phosphatase and by expression of Oct3/4, as discussed above. The RPES cells typically retain their stem cell phenotype when cultured on appropriate feeder cells. However, they can initiate differentiation under a variety of circumstances.

Thus removal from feeder cells, or culture in suspension, followed by replating in the absence of feeder cells in appropriate tissue culture flasks results in differentiation of stem cells into a variety of cell types that include neurons, muscle of various sorts and haematopoietic cells (see descriptions in Robertson 1987a). Differentiation of pluripotent stem cells may also be initiated by altered conditions affecting cell density and aggregation (e.g. seeding at low cell densities or trypsinisation) or by forcing growth suspensions by exposure to various agents that include but are not restricted to retinoic acid, and other retinoids, hexamethylene bisacetamide, and the bone morphogenetic proteins (see Robertson 1987a; Andrews 1984; Andrews et al

1982, 1990, 1994, 1996; Thomson et al 1998). The type of cells that arise depend upon the nature of the inducing agent, and the culture conditions including the presence or absence of specific growth factors or other molecules.

Discussion

Although pluripotent stem cell lines have been derived from early embryos (Robertson, 1987b; Thomson et al 1995, 1998), primordial germ cells (Matsui et al 1992; Shamblott et al 1998) and from germ cell tumours (reviewed, Andrews, 1998) of various species, including the laboratory mouse, rhesus monkeys and humans, and nuclei from differentiated somatic adult cells have been re-programmed to yield embryonic stem cells by transplantation to enucleated oocytes (Campbell et al 1996; Wakayama et al 1998), there are no reports that pluripotent stem cells, resembling embryonic stem cells with the capacity to differentiate into a variety of functional somatic cell types, can be produced by the re-programming of differentiated or committed embryonic or adult somatic cells, or extra-embryonic cells, without the use of oocytes.

We now describe methods by which ES/EG cells can be used to re-program various somatic, differentiated cells, or other embryonic or extra-embryonic cell types, to a state from which they can then be induced to differentiate into one or more functional differentiated cell types that are distinct from the parental cells. In the best cases, but not necessarily in all cases, the re-programmed cells produced by this technique, called 'Re-programmed Embryonic Stem Cells' (RPES cells), resemble embryonic stem cells derived directly from early embryos, and can be induced to differentiate into a broad range of functional, differentiated cell types that include, but are not limited to, neurons, muscle (including skeletal and cardiac muscle) and

haematopoietic cells. These RPES cells are diploid with a normal karyotype, and isogenic with the differentiated parental cells from which they are derived. They may be used to generate differentiated cells for transplantation and use in cell and tissue replacement therapies.

In some cases, only partial reprogramming occurs with, for example, the activation of several genes that are not active in the parental differentiated nuclear donor cell. Such cells are also of use in a variety of these same circumstances.

An example of such a gene is Oct4. Oct4 has previously been reported to be characteristically expressed by undifferentiated EC and ES cells (Brehm *et al.*, 1998). Therefore, to test the ability of human EC cell cytoplasm to reprogram somatic cells, isolated mouse thymocytes were fused with human EC cells, (2102Ep, clone 4D3 (Andrews *et al.*, 1982) or TERA1 (Fogh and Trempe, 1975; Andrews *et al.*, 1980)), to produce heterokaryons which were tested after 2 days for activation of Oct4 expression from the thymocyte genome. Evidence for such activation would indicate, not only that human EC cells are capable of re-programming a somatic cell nucleus to an ES/EC cell like state, but also that the regulatory factors involved are capable of working between different mammalian species. Thus if human EC cells can reprogram a mouse somatic cell, we would anticipate not only that they would be able to reprogram a human somatic cell, but also that mouse EC cells would be able to reprogram human somatic cells as well. Similarly, given the resemblance of EC and ES cells, it would be expected that ES cells could reprogram somatic cells in the same way as EC cells.

In Experiment 1, as anticipated, an amplified band (573 bp), corresponding to human Oct4 expression was detected similarly in RNA preparations from the 2102Ep x thymocyte fusion in the presence of PEG, and in the mock fusion in the absence of PEG, consistent with its expression by 2102Ep human EC cells.

However, a band corresponding to mouse Oct4 (415 bp) was only detected in the RNA preparation from the 2102Ep x thymocyte fusion in the presence of PEG, when heterokaryons were expected to be present. The corresponding absence of mouse Oct4 from the mock fusion indicates both the absence of Oct4 expression from mouse thymocytes in this experiment, and the requirement for formation of heterokaryons for its activation from the thymocyte genome by the 2102Ep cytoplasm. No products were seen in the 'water' control, indicating absence of contamination.

In a second experiment, in which 2102Ep and TERA1 human EC cells were fused with mouse thymocytes in the presence of PEG, mouse Oct4 was only detected in the 2102Ep fusion, again confirming the ability of 2102Ep cells to reprogram mouse thymocytes with activation of Oct4 expression, but suggesting in this experiment that TERA1 cytoplasm did not achieve reprogramming. In both cases, human Oct4 was detected as expected, consistent with its expression by 2102Ep or TERA1 human EC cells.

In further controls, no mouse Oct4 expression was detected in RNA prepared from isolated mouse thymocytes not used for fusion. However, a similar sized PCR band to that detected in the 2102Ep x thymocyte fusion samples, corresponding to mouse Oct4, was detected in mouse PCC4 EC cells as expected.

In our method, RPES cells are created by combining the nucleus from a differentiated or committed cell (the Nuclear donor), whether from adults or from embryos, with the cytoplasm from an ES/EG cell (the Cytoplasm donor), from which the nucleus is removed. Several methods can be used to combine the nucleus from the differentiated cell and the cytoplasm from the ES/EG cell; in some methods the ES/EG cell nucleus is removed prior to

combination of the cytoplasm with the donated nucleus, and in other methods the ES/EG cell nucleus is removed after combination. If ES/EG cells and differentiated cells from the same species are used, then the resulting RPES cells retain cytoplasmic genetic determinants (e.g. the mitochondrial genome) and a nuclear genome from the same species. By contrast, embryonic stem-like cells produced by transplantation of somatic cells into enucleated oocytes of other species will continue to harbour mitochondria of that other species. Especially for the production of human RPES cells and their differentiated derivatives for transplantation into a human host, the maintenance of a human nuclear and human cytoplasmic genome could be a distinct advantage.

The method that we describe incorporates the techniques for maintaining and propagating the RPES cells produced, and the techniques for inducing them to differentiate into a range of differentiated, functional cell types.

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